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Probing WW domains to uncover and refine determinants of specificity in ligand recognition

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Abstract

Understanding the specificity of protein–protein interaction mediated by domains and their ligands will have strong impact on basic and applied research. Visual inspection of WW domain sequences prompted a general classification of the domains into two large subfamilies. One subfamily contains two consecutive aromatic residues in the beta 2 strand of the domain whereas the other contains three or four consecutive aromatic residues in the same position. In the recent past, we proposed a rule of 'two vs. three aromatics' in the beta 2 strand of WW domains as a molecular discriminator between Class I and Class II WW domains, which recognize PPxY or PPLP motifs, respectively. Using phage display libraries expressing WW domains with random sequences replacing a part of the beta 2 strand, we provided additional evidence supporting our rule. We conclude that three consecutive aromatic amino acids within the beta 2 strand of WW domain are required but not always sufficient for the WW domain to belong to Class II.

Introduction

Protein modules are well demarcated and independently folded portions of proteins comprising 40–200 amino acids (Pawson and Scott 1997; Sudol 1998). They are considered as 'Nature's LEGO blocks' used by proteins to mediate protein to protein interactions and to transduce signals (Sudol 1996a). Understanding the specificity of protein–protein interactions mediated by domains and their cognate peptide ligands should have broad ramifications for basic and applied research including optimization of bio-processes relevant to cytotechnology.

The WW domain is one of the smallest protein modules, composed of only 40 amino acids, that folds as a monomer in solution without disulfide bridges or cofactors (Bork and Sudol 1994; Chen and Sudol 1995; Sudol et al. 1995). The name refers to the presence of two conserved tryptophan residues (W), which are spaced 20–22 amino acids apart (Sudol 1996b). The domain was shown to mediate specific protein–protein interactions in plants, yeast, nematodes, flies and mammals (Sudol 2002). Some of the signaling complexes that involve the WW domain have been implicated in human diseases including Muscular Dystrophy, Alzheimer's Disease and a genetic form of Hypertension known as Liddle's Syndrome (Sudol and Hunter 2000; Sudol 2002).

Solution and crystal structures of the WW domain were solved (Macias et al. 1996; Huang et al.

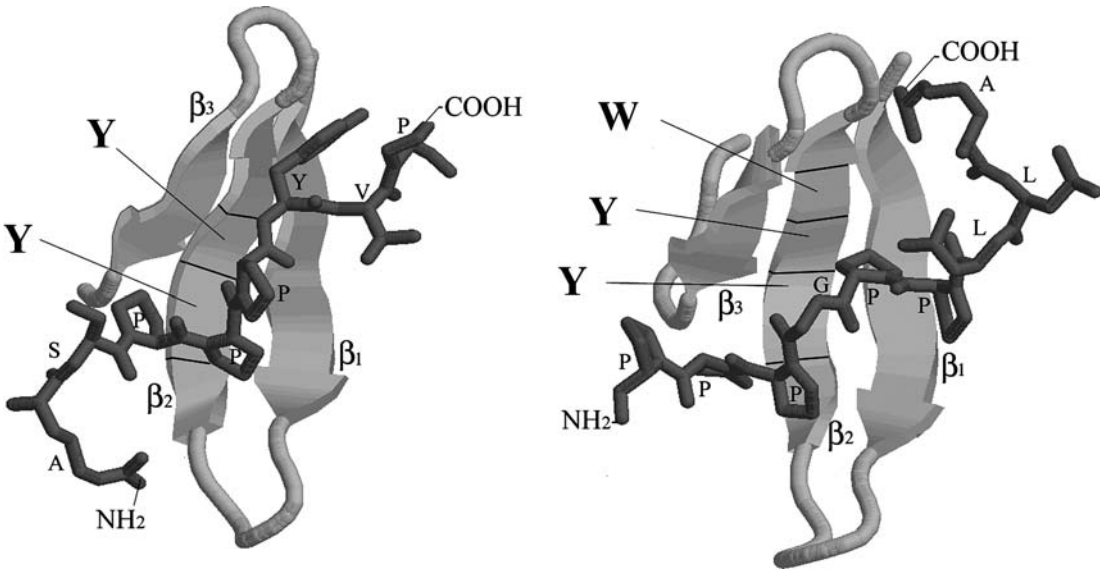


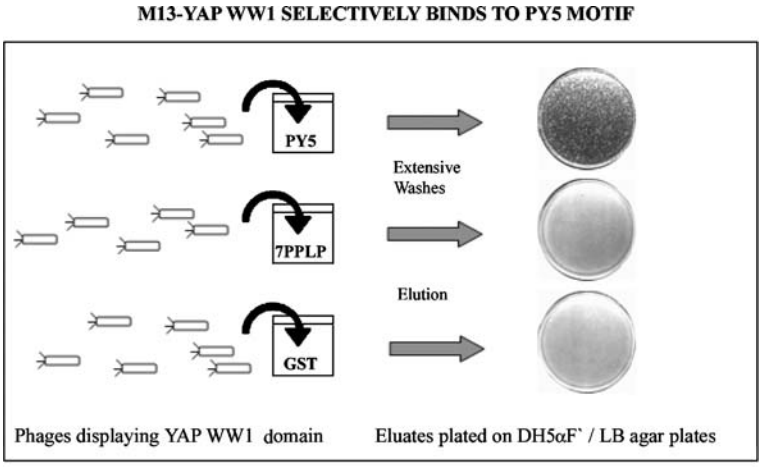
Figure 1. Schematic structures of Class I and Class II WW domains with two and three aromatic residues in the beta 2 strand. See Huang et al. (2000) and Macias et al. (2002) for more details. Left Panel: Class I WW domain of dystrophin binding to the ASPPPYVP fragment of the beta-dystroglycan peptide. Right Panel: Class II WW domain of Npw38/PQBP-1 binding to the PPPGPPLLA peptide.

2000; Wiesner et al. 2002). The hallmark of the WW domain structure is a three-stranded beta-sheet with a hydrophobic pocket for proline-rich ligands (Figure 1). High-resolution crystal structure of the WW domain of dystrophin identified 'aromatic cradle' between the conserved tryptophan of the domain and the required proline of the ligand. Such a molecular arrangement is present in other domains that bind prolines, including Src-Homology 3 (SH3) and Ena Vasp Homology 1(EVH1) domains (Huang et al. 2000; Zarrinpar and Lim 2000).

Based on the ligand recognition there are four classes of WW domains. The first class binds ligands containing PPxY motif. Class II and III recognize proline-rich sequences flanked or interrupted by L or R residues. Class IV is represented only by several WW domains that recognize ligands with phospho-SP or phospho-TP cores in a phospho-dependent manner (Sudol and Hunter 2000; Macias et al. 2002). Class II and III WW domains recognize proline-rich ligands without aromatic residues and both could also be placed in a larger group that recognizes ligands with PPxPP consensus. Certain members of this group seem to possess 'binding pockets' reminiscent of SH3 modules (Macias et al. 2002).

Phage-displayed peptide/polypeptide repertoires are ideal to study ligand predilection of WW domains (Linn et al. 1997; Dalby et al. 2000). Class I WW domains were shown to have a subclass that selects ligands with LPxY cores (Kasanov et al. 2001). Based on the biochemical and structural analysis, WW domains of Class I and Class IV were each further subdivided into two subclasses: Ia, Ib and IVa, IVb (Kato et al. 2002). The major criterion of this classification was the presence or absence of R residues in the beta 1 strand and the loop between the beta 1 and beta 2 strands (see Discussion and Figure 3).

We proposed a rule of 'two vs. three aromatic residues' in the beta 2 strand of WW domain as a molecular discriminator between Class I and Class II WW domains (Sudol 1998; Espanel and Sudol 1999). The proposal came from visual inspection of a dozen of WW domain sequences for which binding data were available. Two WW domains were of special interest because of important signaling functions: Yes-Associated Protein (YAP) that acts as a signaling adaptor for ErbB4 receptor (Komuro et al. 2003) and Franca Esposito clone #65 (FE65) that functions as an adaptor of Amyloid Precursor Protein (Sudol et al. 2001). We also elected to convert the specificity of YAPWW domain that belongs to Class I to that of FE65 that belongs to Class II by generating molecular repertoires in selected amino acid positions, which were shown to be involved



LIBRARIES IN M13

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YAP WW1:QSSFEIPDDVPLPAGWEMAKTSSGQRYFLNHIDQTTTWQDPRKAMLSQMNVTAPTS
YAMI-X4:---------------------------XXXX-----------------------
YAMI-X7:---------------------------XXXXXXX--------------------
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Selected Phages

BAIT		SEQUENCES	AFFINITY	TIMES
	YAP WW1	WEMAKTSSGQRYFLNHIDQTTTW	+++	
	YAMI-X4/7	WEMAKTSSGQRYXXXXXXXTTTW		
PY5	PY4 #1	WEMAKTSSGQRYFISNIDQTTTW	+++	6
	PY4 #2	WEMAKTSSGQRYFLIVIDQTTTW	+++	1
	PY7 #3	WEMAKTSSGQRYFLDRVHRTTTW	+++	7
7-PPLP	PL7 #3	WEMAKTSSGQRYFWRALDGTTTW	+++	1
	PL7 #7	WEMAKTSSGQRYFWLRSSRTTTW	+++	1
	PL7 #4	WEMAKTSSGQRYFYTRDGVTTTW	+	2

Figure 2. Summary of phage display selection for sequences binding to PPxY or PPLP core-containing peptides. Upper panel documents that the phage-expressed YAPWW1 domain recognizes only a PPxY–containing peptide and does not recognize a PPLP-containing peptide. These data verified suitability of our experimental system. YAPWW1 domain was used to generate two libraries, displaying the domain on the surface of the phages: YAMI-X4 and YAMI-X7 with 4 or 7 consecutive residues (see Xs), respectively, substituted by random sequences. The sequence expressed in the phage (middle panel) is longer than the WW domain. Based on the overall sequence similarity among family of WW domains the arbitrary boundaries of the domain were proposed (Bork and Sudol, 1994). The YAP WW domain sequence starts with PLPA... at the amino-terminus and ends with ...PRKA at the carboxy-terminus. Beta strands are as follows: beta strand 1: WEMAKT; beta strand 2: QRYFLNH; and beta strand 3: QTTTW. The relative affinity of the selected phages for peptides was evaluated by visual inspection of the density of peptide-selected phages and by color intensity of the ELISA assay that estimated the level of phage coat protein (Linn et al. 1997). When compared to control wells, where no peptide but only albumin-containing buffer was used, the almost confluent plate with phages and the most intense yellow color in the ELISA assay were given the highest score (+ + +); less than one third plate covered with phage plaques and a pale yellow color that was clearly above the background in ELISA, were given score (+). Readings of ELISA corresponded well to the relative density of phages plated from parallel assay wells.

in ligand binding according to NMR structure (Macias et al. 1996; Espanel and Sudol 1999). Two residues in the beta 2 strand (L and H) and one residue in the beta 3 strand (Q) of GST-YAPWW domain were replaced. These steps generated a repertoire of mutants produced as fusion proteins in bacteria and screened with PPLP core-containing peptide for Class II WW domain binding. Using this approach, we concluded that a single substitution of L to W in the beta 2–5 position (see Aasland et al. 2002 for nomenclature) of YAP was sufficient to convert, at least in part, the binding of Class I WW domain to that of Class II (Espanel and Sudol 1999). Based on this result plus more extensive studies in the same experimental model, we proposed that three consecutive aromatic amino acids within beta 2 strand of a WW domain are

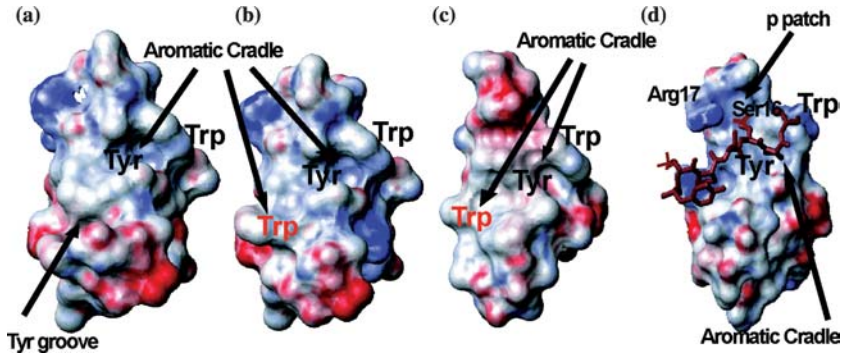

Figure 3. Surface representations of the binding sites of Class I, II and IV WW domains. Residues that are involved in binding are shown. The third Trp residues of Class II WW domains are shown in red. See the text for more details. (a) The ligand binding surface of YAP65 WW domain. (b) The modeled surface of YAMI PL7 #3. (c) The model of Fe65 WW domain. (d) The WW domain of human Pin1 with a phospho-TP ligand.

required, but not always sufficient, for the WW domain to belong to Class II.

Since phage displayed peptide and protein libraries provide a better tool for creation of extensive repertoires than expression, plasmid-based libraries in bacteria, we decided to further test our hypothesis of 'two vs. three aromatics' in beta 2 strand as a determinant of Class I vs. Class II WW domain selectivity, using phage technology. In order to confirm that rule and to investigate other functional substitutions in the beta 2 strand, we generated two phage libraries displaying the entire WW1 domain of human YAP with four or seven consecutive residues as randomized sequences. Libraries were selected on PPxY or PPLP cores containing peptides known to represent cognate ligands for Class I or Class II WW domains, respectively. We have confirmed the 'two vs. three aromatics' rule and pointed to additional substitutions that could be tolerated by functional WW sequences that belong to Class I and Class II.

Materials and methods

M13 constructions

M13-YAP WW1 was obtained by inserting PCR fragment coding for the human WW1 domain of YAP (amino acids 162–217) into XhoI and XbaI sites of gene III of a phage vector mBAX (Sparks et al. 1996). To make YAMI-X4 and -X7 libraries, two restriction sites SpeI and KspI were introduced into the YAP WW1 of M13 vector, without changing the amino acid sequence. Two primers harboring the random nucleotides were synthesized:

SK-X4: TTCCT*ACTAGT*TCTGGTCAGAG ATACNNKNNKNNKNNKATCGATCAGACGA-CAACCTGGCAGGA

SK-X7: TTCCT*ACTAGT*TCTGGTCAGAGAT ACNNKNNKNNKNNKNNKNNKNNKACG-ACAACCTGGCAGGA.

Using Klenow fragment and a third primer partially complementary of the two SK primers (CTT*CCGCGG*GTCCTGCCAGGTTGTCG), double strand DNA were obtained. These fragments were digested by SpeI and KspI and ligated into the M13-YAP WW1 vector at the same sites. Approximately one million clones were obtained for each of the two libraries.

Ligands to the WW domains of Class I and II: GST-PY5 and GST-7-PPLP, respectively, were as previously described (Espanel and Sudol 1999, 2001). Briefly, GST-PY5 contained EYPPYPPP-PYP derived from p53BP2 whereas GST-7-PPLP contained PPPPPPLPPPPPP sequence derived from clone 7 representing a Mena-like ligand of the FE65WW domain (Ermekova et al. 1997).

Folding validation

One to two micrograms of GST-'baits' were coated on 96 wells overnight at 4 °C. Wells were blocked with 1X PBS, 0.5%BSA for 1 h at room temperature. After three washes with 1X PBS, 0.1% tween 20, 0.1% BSA, the M13-YAP WW1 phages were tween 20 incubated with either

GST-PY5, or GST-7PPLP, or GST alone. Thirty minutes later, wells were washed five times with 1X PBS, 0.1% Tween 20, 0.1% BSA (Linn et al. 1997; Kasanov et al. 2001). Phages were eluted with 200 mM glycine-HCl, pH 2.2 for 10 min. Neutralization of the eluates was accomplished with an equal volume of 1 M NaH_2PO_4, pH 7.4. One μl out of 500 μl was incubated with DH5α cells (corresponds to one panning) and plated in top agar as described previously (Linn et al. 1997).

Phage display

One to two micrograms of GST-baits were coated on 96-wells plate, as described above. Three rounds of 'panning' were performed (Linn et al. 1997), then phages were plated and assayed by ELISA using antibody for pVIII-M13 protein (Amersham Bioresources). Positive clones were characterized by the direct sequence analysis of their inserts.

Model construction

Homology modeling was carried out using Swiss-Model (Guex and Peitsch 1997). The detail of the manipulation is followed as in previous report (Kato et al. 2002).

Results and discussion

In order to confirm the rule of 'two vs. three aromatics' and to investigate other functional substitutions in the beta 2 strand of a WW domain, we used the phage display method. We have documented that YAPWW1 domain could be displayed on phages and it is able to maintain its binding activity and specificity by recognizing PPxY but not PPLP core containing ligands (Figure 2, upper panel). Two phage libraries displaying repertoires of the WW1 domain of human YAP were constructed. Following the first aromatic residue (Y) in the beta 2 strand, either 4 or seven consecutive residues were substituted by random sequences (Figure 2). Phages were selected on PPxY- or PPLP- core-containing peptides known to represent cognate ligands of YAPWW1 or FE65WW domain, respectively (Chen and Sudol 1997; Ermekova et al. 1997; Espanel and Sudol 2001). As shown in Figure 2, lower panels, in the beta 2 strand position, all clones that were isolated for PPxY binding (Class I) contained two aromatic amino acids, whereas all clones that were selected for PPLP binding contained three aromatic amino acids. Interestingly the F residue was selected in all the clones, right after the fixed Y. This F is also present in the wild type sequence of YAP suggesting a crucial role in the folding and/or in the binding specificity, perhaps not associated with the Y of the PPxY ligand core but with its prolines (Macias et al. 1996; Huang et al. 2000). The third aromatic position in the 'Class II converted' clones was represented by W or Y residue. The W was also the amino acid selected by all the 'Class II converted' clones in the previous screening using the L substitution in plasmid-based library expressed in bacteria (YAFE-LH; Espanel and Sudol 1999). However, the L to Y substitutions did not allow the PPLP binding in our previous screen (Espanel and Sudol 1999). Interestingly, the clone PL7 #4 has a Y as the third aromatic residue that allows binding to PPLP bait. Even if the interaction is apparently weaker in the ELISA test, this data suggests that other substitutions present in the PL7 #4 clone are important for the binding. More experiments have to be done to evaluate the role of substitutions outside of the aromatic block on binding specificity. Among other substitutions, we noted a predilection for L (as in YAP) and I residues after two aromatics in Class I selection but we were surprised by the lack of H residue in all the selected clones. In the wild type YAPWW1 sequence the H residue located in between beta 2 and beta 3 strands was implicated in the stability of the complex (Macias et al. 1996, 2002).

The rule of 'two vs. three aromatics' represents a simplified 'tool' to predict the molecular function of three-dimensional module from a conserved feature of its linear sequence. However, a more appropriate way to uncover the specificity of protein–protein interaction mediated by a protein domain is to discern conserved and functional features in three-dimensional structures of the domain–ligand complexes. The groove named 'aromatic cradle' for WW and SH3 domain complexes is a good example here (Huang et al. 2000; Sudol and Hunter 2000; Zarrinpar and Lim 2000). The –X-P- (-Xxx-Pro-) segment on the ligands for

these domains is recognized by this groove. It seems that the groove is essential for all WW domains to bind their ligands. In addition, Group I and IV WW domains employ the Y-binding groove and the 'p' patch to bind their own ligands, respectively (Figure 3). The Y residue on PPxY sequence on the Class I ligand is recognized by the Y-binding groove, while the pS/pT residue on pS/pT-P ligand core is accommodated by the 'p' patch, defined as specific amino acids in the loop 1 of WW domain. The 'p' patch shown in Figure 3 contains S16 and R17 among other residues (Kato et al. 2002). Like FE65, the PL7 mutants (Figure 2) showed the preference for three aromatic residues instead of L or I in the position of the third aromatic residue, which suggests that these aromatic residues may be one of the significant parts of another *patch* that evolved to recognize the PPLP ligands. In our model structure, the third Trp residue forms another wide groove, which highly resembles the aromatic cradle (Figure 3). These findings suggest that the Trp residue takes part in the recognition of Pro residues in Class II ligands, such as PL motif or other Pro-rich sequences. More structures of Class II WW domain–ligand complexes need to be solved to better understand the involvement of the block of three aromatics on the ligand binding specificity. It is certain that the structural complexity of WW domain 'binding pockets' will be high. This could be inferred from the recent data showing that WW domains of Prp40, having three aromatics in their beta 2 strands have the ability to interact with ligands containing PPxY and PPxPP motifs, placing these domains in Class I and Class II WW domains (Wiesner et al. 2002).

Three recent advances prompted us to focus on the WW domain as the protein module for which we could understand rules of protein–protein interaction. AxCell Biosciences Corporation located in Newtown, Pennsylvania, USA, elected to generate a protein–protein interaction map of the human proteome at the level of protein modules. The WW domain was chosen as the first module for mapping because of the small size of the domain and well-characterized consensus sequences for ligands. More than 69,000 interactions were found and characterized for 70 human WW domains. Apparent Kd of each interaction was determined. Availability of the mapping data from AxCell (Hu et al. 2004), plus observations that the WW domain family, because of the small size of the domain, could be expressed as large molecular repertoires using phage-display methods (Linn et al. 1997; Dalby et al. 2000; Kasanov et al. 2001) or as polypeptide arrays on SPOT membranes (Chen et al. 1997; Linn et al. 1997; Espanel and Sudol, 2001; Toepert et al. 2001), provide us with data and tools for detailed analysis of this protein module.

Concluding remarks

We hope that many, perhaps all, of the variations in primary and tertiary structures of WW domains, which contribute to the specificity of interaction with cognate ligands will be illuminated in the near future. There is no doubt that understanding of these variations, also known as *epsilon* determinants of specificity (Sudol, 1998; Aasland et al. 2002), for WW domain complexes will have broad ramifications for understanding and predicting protein complexes beyond the WW, SH3 and EVH1 family of modules.

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